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PRINCIPAL INVESTIGATOR: Sharon E. Plon, M.D., Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

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Sharon E. Plon, M.D., Ph.D.

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Baylor College of Medicine
Houston, Texas 77030-3498

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The goal of this project is to further define at a molecular level the human gene products required for the normal G2 checkpoint response. The checkpoint response is a fundamental mechanism by which cells control their cell division cycle after experiencing DNA damage from radiation. This response results in an arrest in the G2 phase of the cycle until damage is repaired. We are exploiting the conservation of this pathway among eukaryotes to isolate human checkpoint genes by large-scale complementation screens isolating novel human cDNAs which can complement yeast G2 checkpoint mutant strains. In the first year of this award we have identified several new human cDNAs which are potential novel human checkpoint genes. In other Technical Objectives we have developed a panel of reagents from normal and malignant human breast cancer cell lines to determine the structure and expression of these genes in both normal and malignant mammary cells.

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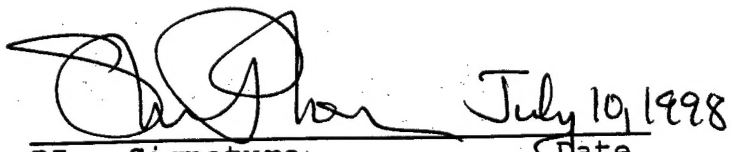

PI - Signature

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Date

Molecular Basis of the Response to Radiation Therapy

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A. Introduction

The goal of this project is to further define at a molecular level the human gene products required for the normal G2 checkpoint response. The checkpoint response is a fundamental mechanism by which cells control their cell division cycle after experiencing DNA damage from radiation. This response results in an arrest in the G2 phase of the cycle until damage is repaired. This checkpoint response is conserved among eukaryotes including the budding yeast *Saccharomyces cerevisiae.* In our application, we proposed to exploit this conservation to isolate human checkpoint genes by large-scale complementation screens isolating novel human cDNAs which can complement yeast G2 checkpoint mutant strains. Subsequent Technical Objectives are directed towards understanding the structure and expression of these genes in both normal and malignant mammary cells including human cell lines and murine models of mammary tumorigenesis. We also planned to perform functional assays of these cDNAs in checkpoint deficient cell lines including the MCF-7 human breast cancer cell line. In this report we detail progress in the first year of this award towards all three objectives.

B. Progress toward completing the proposed Technical Objectives.

Materials and Methods:

Complementation Assay: A *rad9,cdc9-8* strain was grown on YEPD plates. Exponential cultures grown in YM-1 media were transformed with purified cDNA library DNA using a modified Li-Acetate transformation protocol of Schiestl, and Giets and yeast total RNA and denatured salmon sperm DNA as carrier to achieve a transformation efficiency of 300,000 colonies per microgram plasmid DNA. After transformation the plates were incubated at 23°C overnight. In the morning plates were transferred to 30°C and incubated for 5 days. Colonies growing at this point are isolated and retreaked on leucine deficient media at 30°C for further analysis.

Two-hybrid Reagents: Reagents used in the two-hybrid screening include the Gal4-activation domain (AD) library, the Gal4-DNA binding (DB) vector (pPC97), the yeast host strain MV103 (Mat **a**, *leu2*, *trp1*, *his3*, Gal1:HIS3, Gal1:LacZ, Spal:URA3), and 5 constructs in MV103 for use as reference controls during screening (22, 23). Control plasmids include 1) DB-pPC97+AD-pPC86, 2) DB-pRb+AD-E2F1, 3) DB-Fos+AD-Jun, 4) Gal4+AD, 5) DB-dDP-1+AD-dE2F.

Construction of the DNA Binding-Atm Fusion ("bait"): The kinase domain of the human *ATM* cDNA (12) has been cloned into pPC97 (Leu+) *GAL4* DNA-binding domain fusion vector (22, 23). The ability of the pPC97 *ATM* construct to encode the functional Atm protein was tested in yeast by its ability to complement a *mec1* mutant yeast strain. After transformation of the construct and it's vector control, cells were spread onto plates containing leucine-deficient media. Individual colonies were selected and resistance to DNA damage was measured after exposure to UV radiation in a Spectrolinker irradiator.

Activation domain-cDNA library: A human T-lymphocyte cDNA fusion library in the activation domain vector pPC86 (Trp+) was kindly provided by J. La Baer (MGH Cancer Center). The cDNAs were cloned into the *EcoRI* (5') and *SpeI* (3') sites. This library has

approximately $2X10^6$ clones and the average insert size is 1kb. *Atm* is highly expressed in T-lymphocytes and the library appears to have a broad range of cDNAs and has been successfully used in two-hybrid screening (Vidal M. et al unpublished). This library was amplified once by electroporation using electrocompetent *E. coli*, JS4 cells (BioRad, Hercules, CA) followed by replica plating onto LB+Ampicillin plates. The DNA was prepared using a Plasmid Maxi kit from Qiagen.

Selection of *Atm* interacting genes: The bait (Leu+) and the library plasmid (Trp+) were sequentially transformed into the yeast host strain MV103 . The transformants containing the bait and library plasmids were selected on media lacking leucine and tryptophan. Three separate pools of library DNA were used to transform the MV103+pPC97 *ATM* cells and 500,000 transformants from each pool were obtained.

The two-hybrid screen was performed by first selecting for growth of Atm bait-library co-transformants on Sc-His-Leu-Trp+25mM 3AT. Subsequently additional reporter genes, *URA3* and *LacZ*, were selected for in the 3AT positive clones. The expression of the *URA3* gene was both selected for on media lacking uracil as well as counterselected against on media containing uracil and 0.1% 5-fluoroorotic acid (5FOA). Induction of the *LacZ* gene was assayed qualitatively in the presence of X-Gal for blue colonies. The phenotypes were then scored. Clones positive for all the reporters were PCR cloned into a pPCRII vector using TA-cloning kit from Invitrogen (Torry Pines, CA). Both strands of the DNA were then sequenced using a LI-COR automated sequencer.

Cell Cultures, Transfection and Expression Vectors: A panel of breast cancer cell lines including, MCF7, MDA-MB-157, MDA-MB-231, MDA-MB-136, BT-20, HBL100, SKBR-3 (all from ATCC) and normal mammary epithelial cells, H-MEC (Clonetics Corp, San Diego, CA) were maintained under similar conditions in DFCI media as previously described. Cells including the MCF7 cell line were transfected with appropriate plasmids in 100-mm dishes by the calcium phosphate method. A fixed amount of plasmid DNA was used in any given experiment. The amount of expression vectors was normalized by adding blank vectors to control for the promoter competition effect. When necessary, transfection efficiency was monitored by use of 1ug CMV-βgal plasmid per transfection, and calorimetric β-gal assay were performed using ONPG as a substrate.

Extraction of DNA and RNA, and Northern Analysis: Total RNA was extracted from breast cancer cell lines using the Qiagen kit. DNAse-I treated RNAs were Northern blotted from 1% formaldehyde gels with Hybond N+ sheet (Amersham). Human GADPH, a house keeping gene was used as a control to access equal loading. The probes were hybridized overnight at 65^0C in 10% dextran sulphate, 2xSSC, 1%SDS, and 250μg/ml salmon sperm DNA. The final wash was in 0.1XSSC-0.1%SDS at 65^0C. Quantitation of the Northern blots was done by detection on an Image Quant phosphoimager.

RESULTS

1. Technical Objective 1 - Isolation of additional human G2 checkpoint genes.

a. We have carried out substantial screening by complementation of a yeast strain mutated in the *RAD9* checkpoint gene. Screening was performed by transformation of a *rad9,cdc9-8* double mutant yeast strain with a human cDNA library cloned into a yeast expression vector. After transformation plates are placed at 30°C and incubated for five days. Approximately 400,000 transformants were screened and 15 colonies growing at 30°C were isolated. These isolates were then further characterized for resistance to DNA damage. Plasmids were rescued from each of these 15 transformants by glass bead lysis and transformed into *E.* coli for isolation. These human cDNA plasmids were then re-transformed into yeast and plated at 30°C in order to determine plasmid dependence of the phenotype. One of these 15 human cDNAs (tx5a) gave reproducible complementation of the *rad9* checkpoint defect. The tx5a cDNA contains an insert of 1kB in length and sequencing is underway of this clone. More extensive screening of this library will be carried out in year two of this grant.

b. With the availability of human cDNA sequences from databases of anonymous cDNAs we have also searched for human G2 checkpoint genes by computer search. Analysis of cDNA databases suggests the occurrence of human cDNAs with at least partial homology to *RAD17, RAD24, RAD9* and *DUN1*. However, the amount of sequence data on these anonymous cDNAs is limited. To date we have obtained cDNA samples for the *RAD9, RAD17 and RAD24* homologs. Additional sequencing of these six anonymous cDNA constructs did not lead to a strengthening of the homology but suggested that they may only contain a few conserved domains. Despite this negative finding the exponential growth in human cDNA sequence available to researchers suggests that this will be a very fruitful route to obtaining additional human G2 checkpoint genes and we are continuing to refine our search strategies. In particular the development of full-length cDNA databases will facilitate this methodology. We expect that over the next year a substantial effort will be expended on identification of human checkpoint genes by homology search.

c. A second genetic screen of human cDNAs in yeast was performed using the human G2 checkpoint gene *ATM*. We performed an extensive screening of human cDNAs which would interact with the human *ATM* protein product. This was performed by generation of a "bait" construct which contains the protein kinase domain of Atm. This construct was then shown to encode a functional protein in yeast and to not have high background in the assay. A human cDNA library expressed in yeast was then screened (approximately one million cDNAs) for human cDNAs which encode proteins capable of interacting with the kinase domain of Atm. This resulted in the isolation of two novel human genes which had not been previously characterized. One of these genes is homologous to a large set of proteins from plants which are involved in the response to stress. We are continuing a thorough analysis of this gene including it's chromosomal localization, expression pattern in normal and malignant tissues and it's role in the response to DNA damage.

2. Technical Objective 2A – Checkpoint gene structure and expression in human breast cancer cell lines.

As described in the original grant application we obtained normal human mammary epithelial cells (HMEC) from Clonetics and a panel of eight human mammary derived cell lines, MCF10A, MCF7, MDA-MB-157, MDA-MD-231, MDA-MB-136, BT-20, HBL100 and SKBR-3. These were all grown in culture under controlled conditions. All the cell lines were grown in the same DFCI media to minimize artefacts due to culture technique including both the immortal but not transformed MCF10 and the transformed lines. One exception is the HMEC cultures which require a separate proprietary media. In parallel RNA, DNA and protein lysates were derived from these cultures. The isolation of RNA was repeated on a fresh set of cultures in order to be able to replicate any findings on the first set.

Over the year, we have used these reagents extensively. CDNAs isolated in the original complementation screens (human *CDC34* and *CHES1**) have been analyzed for their expression pattern in these mammary lines. To date, we have performed Northern blot analysis most extensively. *CDC34* is expressed in all cell lines tested including normal HMEC cultures and malignant lines. *CHES1* only hybridizes very weakly to the Northern blots. This will require development of a RT-PCR or RNAse protection assay in order to have sufficient sensitivity. As antibodies for these proteins become available we will also use the protein lysates to look at protein expression.

3. Technical Objective 3 - Determination of Changes in Response to Radiation of a Human Breast Cancer Cell Line upon Expression of Human Checkpoint Genes.

The first portion of this aim is based on examining whether the human cDNAs previously identified or identified in Aim 1 will suppress the G2 checkpoint defect in the human MCF-7 breast cancer cell line. This aim has been completed for the *CHES1* cDNA. We have expressed *CHES1* in MCF-7 cells both by transient transfection and by infection with a *CHES1* recombinant adenovirus. After infection we have examined changes in the exponential growth of the cells as well as their response to DNA damage. To date we have not detected any change in this behavior compared with control vectors.

The same methodologies will be used to examine the effect of expression of other cDNAs in the MCF-7 line. In particular, a great deal of effort was expended to optimize the transient transfection assay. This is performed by co-transfecting with a CD20 plasmid and then performing FACS analysis where the transfected cells are gated on CD20 content and simultaneously stained with propidium iodide. This technique should now allow us to rapidly look at the effect of the other cDNAs without the need to construct a recombinant adenovirus or retrovirus for each cDNA. The use of the transient transfection technique has several advantages over construction of recombinant retrovirus (as originally proposed in the application). In particular, it is much more rapid and will allow analysis of the effect of several different human cDNAs to be assayed in parallel.

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* Note that the human cDNA originally identified in the application as *CCC1* was renamed *CHES1* (for checkpoint suppressor - 1) prior to publication in order to avoid confusion with an unrelated yeast gene simultaneously named *CCC1*.

C. Conclusions

In the first year of this four-year Career Development Award the PI has made substantial progress towards all three Technical Objectives. These results include isolation of a human cDNA which can partially suppress the yeast *rad9* defect and two novel human cDNAs which can interact with the human checkpoint protein ATM. In addition, methodologies to isolate additional homologs are in place, in particular with an added emphasis on isolation by identification of homologous cDNAs in human cDNA databases.

The subsequent objectives are focused on determination of whether cDNAs isolated in genetic screens are altered in expression or structure in breast cancers. The reagents including RNA, DNA and protein from human breast cancer cell lines grown under identical culture conditions have been produced. This set of reagents will now be available for studies in future years of this award as additional cDNAs are isolated.

In objective 3 we proposed to determine if any cDNAs isolated in these genetic screens are capable of altering the response to radiation treatment of the human breast cancer cell line MCF-7. The methodology to answer this question rapidly by use of a transient transfection assay has been developed in the laboratory. This question has been answered in the negative for the human *CHES1* cDNA. Optimization of this complex protocol will allow rapid checkpoint determinations for human cDNAs identified in subsequent years of the grant.

Statement of Work
The following Tasks have been accomplished
Technical Objective 1
Task 1
Task 2 – for *rad9* strains
Task 3
Technical Objective 2
Task 1
Task 2
Technical Objective 3
Task 1 (use of an adenoviral vector was chosen over retroviral vector)
Task 2
Task 3

The original application proposed to perform the three Technical Objectives in a roughly sequential pattern. However, in practice we have found it more efficient to begin work on all three aims in a parallel fashion. Thus, we have completed portions of Technical Objective 2A which were originally predicted to occur in months 14-18 and portions of Technical Objective 3 slotted for months 20-36. Development of these reagents earlier in the award period should allow for more efficient analysis of clones obtained in Technical Objective 1. Given the increasing availability of human cDNA databases we expect to extend the search for human homologs described in Technical Objective 1 throughout the second year of this award.

D. References

Results of this work have been presented at the following meetings.

AT Children's Project National Meeting - August 1997, Baltimore, Maryland. Invited Oral Presentation and Poster

ALTERNATIVE DNA DAMAGE CHECKPOINT PATHWAYS IN EUKARYOTES

Sharon E. Plon, Debananda Pati, and Yi Chen J. Li. Texas Children's Cancer Center, Department of Pediatrics, Baylor College of Medicine, Houston, Texas

Genetics Society of America - DNA Repair: From Bacteria to Humans – March 1998 - Airlie Virginia. Poster Presentation

ALTERNATIVE DNA DAMAGE CHECKPOINT PATHWAYS IN EUKARYOTES

Sharon E. Plon, Debananda Pati, Yolanda Sanchez, and Stephen Elledge. Departments of Pediatrics and Biochemistry, Baylor College of Medicine, Houston, Texas